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ORIGINAL ARTICLE

TGF-β1 promotes epithelial-to-mesenchymal transition and stemness of prostate cancer cells by inducing PCBP1 degradation and alternative splicing of CD44

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Abstract

CD44 is a marker of cancer stem cell (CSC) in many types of tumors. Alternative splicing of its 20 exons generates various CD44 isoforms that have different tissue specific expression and functions, including the CD44 standard isoform (CD44s) encoded by the constant exons and the CD44 variant isoforms (CD44v) with variant exon insertions. Switching between the CD44v and CD44s isoforms plays pivotal roles in tumor progression. Here we reported a novel mechanism of CD44 alternative splicing induced by TGF-β1 and its connection to enhanced epithelial-to-mesenchymal transition (EMT) and stemness in human prostate cancer cells. TGF-β1 treatment increased the expression of CD44s and N-cadherin while decreased the expression of CD44v and E-cadherin in DU-145 prostate cancer cells. Other EMT markers and cancer stem cell markers were also upregulated after TGF-β1 treatment. RNAi knockdown of CD44 reversed the phenotype, which could be rescued by overexpressing CD44s but not CD44v, indicating the alternatively spliced isoform CD44s mediated the activity of TGF-β1 treatment. Mechanistically, TGF-β1 treatment induced the phosphorylation, poly-ubiquitination, and degradation of PCBP1, a well-characterized RNA binding protein known to regulate CD44 splicing. RNAi knockdown of PCBP1 was able to mimic TGF-β1 treatment to increase the expression of CD44s, as well as the EMT and cancer stem cell markers. In vitro and in vivo experiments were performed to show that CD44s promoted prostate cancer cell migration, invasion, and tumor initiation. Taken together, we defined a mechanism by which TGF-β1 induces CD44 alternative splicing and promotes prostate cancer progression.

Keywords CD44 · Epithelial-to-mesenchymal transition · Alternative splicing · Cancer stem cell

Introduction

Epithelial-to-mesenchymal transition (EMT) is a tightly regulated process that plays critical roles in tumor progression [1]. EMT enhances the migratory and invasive property of cancer cells, and directly links to the generation of stem cell-like tumor-initiating cells [1, 2]. CD44, a multifunctional transmembrane protein, is a marker of CSC, and has been found to be involved in many types of tumors [3–5]. At the molecular level, high CD44 expression and CD44 alternative splicing were reported to correlate with EMT and the generation of cancer stem cells [3–5].

CD44 was originally characterized as a receptor for hyaluronic acid (HA) and lymphocyte-homing receptors. Once binding to HA or lymphocyte-homing receptors, CD44 molecules are oligomerized to activate downstream signaling events and transcription programs, which mediates

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the physiological roles of CD44 in cell adhesion and cell migration [6, 7]. Due to the similarities between tumor metastasis and lymphocyte homing and migration, CD44 has been studied in tumor biology for decades.

The human CD44 gene consists of 20 exons. Alternative splicing of these 20 exons generates 19 CD44 mRNA variants [8]. The smallest variant, called the standard isoform of CD44 (CD44s), contains the constant exons 1–5 and 16–20 [8]. The exons from 6 to 15 are variant exons (V1–V10), which can be inserted into the constant exons at different combinations by alternative splicing to form many different types of CD44 variants (CD44v) [8]. CD44s, also known as the mesenchymal isoform, are ubiquitously expressed in many tissue and cell types. While CD44v is mainly expressed in epithelial cells, tumor cells or cells related to inflammation, it is also called the epithelial isoform (CD44E) [8]. It is well-documented that the expression of CD44v is suppressed and the expression of CD44s is upregulated during EMT, which is a key molecular mechanism controlling the process of EMT [9, 10]. TGF-β1 is a strong inducer of EMT and it plays important roles in tumor progression [11] [12]. We and others have reported that TGF-β1 treatment increased the expression of CD44s and suppressed the expression of CD44v in prostate cancer cells, and they adopted potent migration, invasion, and tumor initiation capacities [13]. A switching from CD44v to CD44s is also reported to be essential for EMT in many other types of tumor cells, including breast cancer [9], liver cancer [14], and ovarian cancer cells [15].

Different splicing factors may be involved in regulating CD44 mRNA alternative splicing in different types of tumor cells. For example, ESRP1 and ESRP2 mediated the v8–v10 and v6–v10 alternative splicing in breast cancer cells and Hela cells [9, 16, 17]; U2AF2 expression was correlated with the switching between CD44v and CD44s in melanoma tumor cells [18]; Tra2β and SRm160 was involved in CD44 alternative splicing in gastric cancer cells [19, 20]. Our previous report found PCBP1 was able to regulate CD44 alternative splicing in prostate cancer cells, which subsequently enhanced their tumor initiation and invasion capacity [13]. The role of PCPB1 in mediating CD44 alternative splicing was also reported in Hela and breast cancer cells [21] . In this paper, we further study the important role of CD44 splicing in prostate cancer cells and begin to dissect the underlying mechanism. We found that TGF-β1 treatment led to increased phosphorylation and degradation of PCBP1, which in turn suppressed alternative splicing of CD44 and mediated a switching from CD44v to CD44s. The elevated expression of CD44s promoted EMT and upregulated stem cell markers on prostate cancer cells, and subsequently rendered them enhanced invasion and tumor initiation capacities.

Materials and methods

Reagents

RPMI1640 (catalog No. A1049101), DMEM (catalog No.10569044), fetal bovine serum (FBS, catalog No. 10099133), and TRIZOL reagent (catalog No.15596018) were purchased from Thermo Fisher. DRB (5,6-dichlorobenzimidazole 1β-d-ribofuranoside, catalog No. D1916) was purchased from Sigma. ProLong Gold anti-fade reagent with DAPI wereurchased from Thermo Fisher. MEK1/2 inhibitor U0126 (catalog No. HY-12031), CDK inhibitor flavopiridol (Catalog No. HY-10005), and AKT inhibitor VIII (Catalog No. HY-10355) were purchased from MedChemExpress.

Plasmids and lentivirus production

CD44s and CD44v6 RNAi-resistant expression plasmid, and shRNA expression plasmid were purchased from Genechem Company (Shanghai, China). shRNA targeting sequences were listed below. ShCD44: Sense: 5'-GCU CCACCUGAAGAAGAUUGU-3'; Antisense: 5'-ACA AUCUUCUUCAGGUGGAGC-3'; ShSMAD3: Sense: 5'-GCCAUCCAUGACUGUGGAUUU-3'; Antisense: 5'-AUCCACAGUCAUGGAUGGCUU-3'; ShPCBP1: Sense: 5'-GGAAGUAGGA AGCAUCAUUUU-3'; Antisense: 5'-AAUGAUGCUUCCUACUUCCUU-3'; ShNC: Sense: 5'- GUACUUUUGUGUAGUACAAUU-3'; Antisense: 5'-UUGUACUACACAAAAGUACUU-3'. Plasmids were transfected into cells using FuGENE HD transfection reagent (Roche, 4709705001). Lentivirus expressing shRNA was produced in 293T cells as previously described [22].

Cell culture

Cells were purchased from American Type Culture Collection (Manassas, VA, USA). DU-145, LAPC-9, LNCaP, LAPC-4 cells were cultured in RPMI1640 containing 10% FBS. Normal human prostate epithelial cell RWPE-1 was cultured in high glucose DMEM containing 5% FBS.

For analyzing CD44 variant expression by quantitative PCR, cells were treated with 100 μM DRB for 4 h, washed with PBS, and then cultured in freshly prepared serum free medium containing 5 ng/ml TGF-β1 for 14 days.

DU-145 cells stably expressed CD44 shRNA or control shRNA were established by puromycin selection as previously suggested [23].

RNA extraction and real time PCR

Total RNA from collected cells or tissue samples were extracted using Trizol reagent according to the methods provided by the manufacturer. cDNA was synthesized using the MMLV Reverse Transcriptase (catalog No. 639522) from TaKaRa Bio. Quantitative real-time PCR was performed in the ABI 7500 fast system using the PCR amplification kit (catalog No. RR420A) from TaKaRa Bio. The data were analyzed using the $2^{-\Delta\Delta CT}$ method [24] and normalized to GAPDH. PCR primers for specific genes are listed in Table S1.

Western blot

Cells were collected and lysed in Cell Lysis Solution (Sigma-Aldrich, St. Louis, MO, USA), centrifuged at 4 °C, 1000 rpm for 5 min. The supernatant was collected, mixed with 3 × SDS sample buffer, and boiled for 5 min. The protein samples were resolved by SDS-PAGE, and transferred to PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked in blocking buffer (TBS containing 0.1% Tween 20 and 5% non-fat dried milk) for 2 h at room temperature, and then incubated with primary antibodies listed in Table S2 overnight at 4 °C. HRP conjugated secondary antibody was added and incubated for 1 h at room temperature after washing. Finally, the membrane was reacted with ECL detection reagent (Perkin-Elmer Inc.) and the chemiluminiscence was detected using Imagequant LAS4000 (GE Healthcare, Japan).

Immunofluorescence staining

Cells were harvested and fixed in 4% paraformaldehyde, washed with PBS, and permeabilized with 0.5% Triton X-100 in PBS) for 5 min. For actin stress fiber staining, cells were incubated with phalloidin (Multi Sciences) in dark at room temperature for 30 min, counter stained with DAPI at room temperature for 10 min. After washing three times with PBS, labeled cells were mounted on slides and images were captured using confocal microscope. For E-cadherin staining, cells were incubated with blocking buffer containing 10% goat serum (Invitrogen) for 30 min, then incubated with anti E-cadherin (CST, 14472, 1:500) for 1 h at room temperature. The sections were then stained with secondary antibody (488 nm anti-mouse secondary antibody, 1:2000 dilution, CST, 4408) diluted in blocking buffer for 1 h at room temperature. The sections probed with only secondary antibody were served as negative control. Sections were immersed with mounting medium (ProLong Gold anti-fade reagent with DAPI, Invitrogen) to visualize the nuclei. Sections, after stained with fluorescence-labeled secondary antibody, were imaged using confocal microscopy (LSM780 upright, Zeiss).

Flow cytometry analysis

Cells were trypsinized with 0.25% trypsin-EDTA, washed with cold PBS twice, centrifuged at 1000 g for 5 min to collected cells. Cells were resuspended in cold PBS at a final concentration of 1×10^6 cells/ml. For each 100 µl cell suspension, 2 µl c-MET-FITC (eBioscience,11-8858-42) and 2µl CD44-PE (eBioscience, 12-0441-83) were added, mixed and incubated at 4 °C in dark for 30 min. Isotype IgG (Invitrogen, 02-6502) was used as negative control. Cells were run through the Becton Dickinson flow cytometry and the collected data were analyzed by the Cell Quest software.

Immunoprecipitation and ubiquitination analysis

DU-145 cells were treated with TGF-β1, MEK1/2 inhibitor U012, CDK inhibitor flavopiridol, or AKT inhibitor VIII, in the presence or absence of proteasome inhibitor MG-132. Cells were lysed, incubated with protein A/G conjugated beads (Thermo Fisher, 88802), and PCBP1 rabbit monoclonal antibody (Abcam, ab168378) or rabbit IgG (CST, 2729) negative control overnight at 4 °C. The beads were collected by centrifugation. After washing, the bound proteins were eluted and resolved by SDS-PAGE, transferred to PVDF membrane, and detected using anti-Ubiquitin monoclonal antibody according to the western blot method described above.

Cell proliferation assay

Cell proliferation was measured with the Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, CD44 knock-down DU-145 cells were transfected with CD44s or CD44v expression plasmids. Cells were seeded into 96-well plates at the density of 2000 cells/well, cultured overnight, and 10 µl CCK-8 was added into each well of the plates at 0, 24, 48, and 72h after seeding. OD450 was read using the Epoch Microplate Spectrophotometer (Bio Tek, Winooski, VT, USA).

Cell migration and invasion assay

CD44 knock-down DU-145 cells were transfected with CD44s or CD44v expression plasmids. 30,000 cells were seeded into each well of 24-well transwell filters (Corning, NY, USA) with or without matrigel (BD Biosciences, San Jose, CA, USA) covered. 0.6 ml medium supplemented with 10% FBS was added into the lower chamber. Cells were cultured for 12 h for migration assay, and 24 h for

invasion assay. Cell were fixed and stained with 0.1% crystal violet (Richard-Allan Scientific, San Diego, CA, USA) and counted under the microscope.

Xenograft mouse model and in vivo metastasis mouse model

All the experimental procedures were approved by the Institutional Animal Care and Use Committee (protocol number: SH9H-2019-T181-2), and all the experimental procedures were carried on according to the Principles of Laboratory Animal Care approved by National Society for Medical Research. Five-week old nude mice were randomly divided into four groups: shNC+vector, shRNA CD44, shRNA CD44+CD44s, and shRNA CD44+CD44v (n=5 per group). For xenograft mouse model, 5×10^6 cells were injected into the right flank of nude mice subcutaneously. Tumor volume (longer diameter × shorter diameter^2/2) was measured every 3 days. The mice were executed at 33 days after inoculation, tumors were excised and weighted. For in vivo metastasis mouse model, 2×10^6 cells were injected into the lateral tail vein of nude mice (m = 5 per group). After eight weeks, the mice were sacrificed, the lungs were collected, and the number of metastasis foci was counted under a dissection microscope. The tissues were fixed with 4% paraformaldehyde and paraffin sections were prepared. The lung metastases were observed by HE staining.

Patients and tissue samples

This study has been approved by the ethics committee of Shanghai Ninth People's Hospital (Shanghai, China). Thirty patients with prostate cancer were enrolled in this study after written informed consent was obtained (Table S3). The samples included 10 cases of clinical Grade I, 10 cases of clinical Grade II, and 10 cases of clinical Grade III. Adjacent normal tissues were also collected.

Statistical analyses

Statistical analyses were carried out using the SPSS software (version 17.0, Chicago, IL). Student's *t* test was used to compare the means of two population, one-way ANOVA test was used to compare the means of more than two populations. The correlation between the expression of N-cadherin and CD44 mRNA was analyzed by Spearman rank correlation test. All data are presented as the mean ± SD from three independent repeats. A two-sided p value less than 0.05 was considered to be statistically significant.

Results

We first quantified the expression of CD44 isoforms in normal prostate epithelial cells and prostate cancer cells by real-time PCR. The DU-145 prostate cancer cell expressed the lowest level of CD44s and the highest level of CD44v (Fig. 1a), we therefore choose this cell line for the following experiments throughout the paper. The DU-145 cells were treated with 100 μM DRB for 4 h to exclude the effects of RNA transcription, and then treated with 5 ng/ ml TGF-β1, cells were collected at various times after treatment to examine the expression of CD44 variants and EMT markers by western blot. As shown in Fig. 1b, CD44v protein level began to decrease at 4 days after treatment, and it was continuously decreasing until below the detection limit on day 12 after treatment. On the contrary, CD44s protein level becomes detectable at 4 days after TGF-β1 treatment, and it steadily increased throughout the period of experiment. The changing pattern of the EMT marker expression was correlative to that of CD44. The epithelial cell marker E-cadherin began to decrease at 4 or 6 days after treatment and it disappeared at 8 days after treatment. On the other hand, the mesenchymal type marker N-cadherin becomes detectable on day 8 and continuously increased throughout the period of the experiment. The data clearly indicated a correlation between CD44 isoform switching and the induction of EMT. The upregulation of N-cadherin was about 4 days behind CD44s expression, suggesting CD44s expression was important for EMT progression after TGF-β1 treatment in DU-145 prostate cancer cells. Similar results were obtained when the cells were not treated with DRB before TGF-β1 exposure (Fig. S1). Thus, DRB was not used in the following experiments.

To test this idea, we knocked down CD44 expression by shRNA and then tested its effect on TGF-β1 treatment-induced phenotypes. As shown in Fig. 2a and b TGF-β1 treatment induced a switching from CD44v to CD44s expression in DU-145 cells, as indicated by the upregulation of CD44s mRNA and protein levels and the downregulation of CD44v mRNA and protein levels. The CD44-specific shRNA effectively reduced the expression of CD44s and CD44v, while the negative control shRNA did not have an effect. As expected, TGF-β1 treatment suppressed the expression of epithelial makers including γ-catenin, occuludin, and E-cadherin, and it significantly induced the expression of mesenchymal markers vimentin and N-cadherin. Inhibiting of CD44 expression by RNAi knockdown remarkably reversed the expression of the epithelial and mesenchymal markers. We also examined morphological change of the cytoskeleton by confocal microscopy after staining the E-cadherin and the actin fibers.

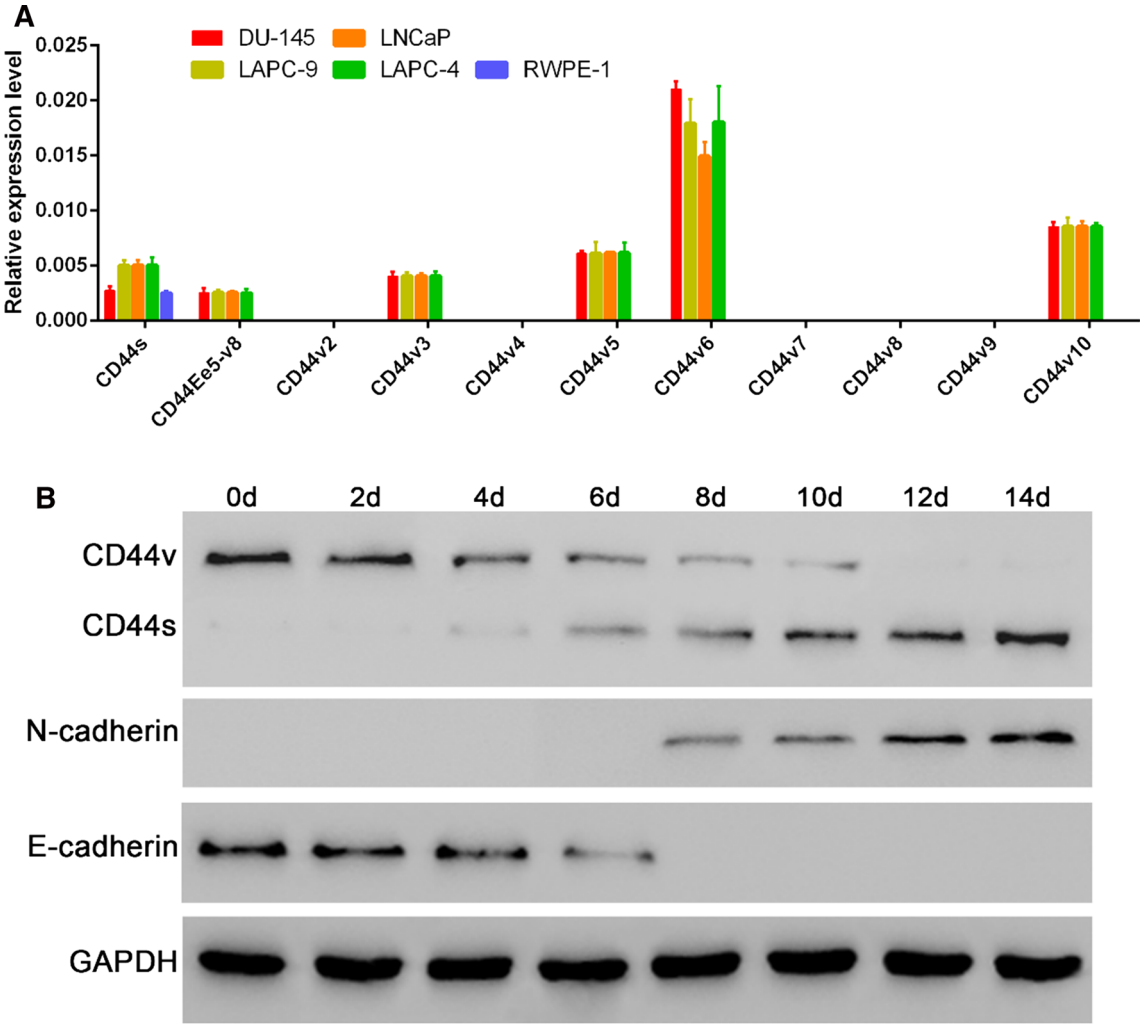


Fig. 1 TGF-β1 treatment induced CD44 isoform switching in prostate cancer cells. **a** mRNA expression of CD44 isoforms was measured by real time-PCR in prostate epithelial cells and prostate cancer cells before TGF-β1 treatment. DU-145 cells expressed the highest level of CD44v and the lowest level of CD44s mRNA. **b** DU-145 cells were treated with 100 μM DRB for 4 h, and then treated with 5ng/ml of TGF-β1. Cells were collected at the indicated days after treatment, and the protein expression of CD44v, CD44s, N-cadherin, and E-cadherin were measured by western blot. GAPDH was used as loading control. CD44s expression was upregulated and CD44v expression was downregulated after treatment. The expression of EMT markers N- and E-cadherin was also changed accordingly

The downregulation of E-cadherin by TGF-β1 treatment expression was observed by immunofluoresence staining and imaging (Fig 2c). TGF-β1 treatment also led to the formation of actin stress fibers, another marker of EMT, as revealed by phalloidin staining. CD44 shRNA treatment clearly reversed the phenotype (Fig. 2c). EMT was known to directly generate stem cell-like tumor-initiating cells. We tested it using flow cytometry analysis after staining the stem cell marker c-Met. As shown in Fig. 2d, TGF-β1 treatment greatly increased the percentage of CD44 and c-Met double positive stem cells from around 1% to over 25%. And again, RNAi knockdown of CD44 in TGF-β1-induced cells reversed that to 1%, similar to the cells without TGF-β1 treatment. Overexpression of CD44s also induced EMT (Fig. S2). All these data indicated that the expression of CD44, particularly CD44s, was essential for TGF-β1-induced EMT and stem cell generation in prostate cancer cells. To further support this conclusion, we tried to rescue the expression of CD44s in RNAi knockdown cells, and then examined the TGF-β1 treatment-induced phenotypes. As shown in Fig. 3, rescue the expression of CD44s but not CD44v6 was able to recapitulate the TGF-β1 treatment-induced EMT and stem cell marker expression in DU-145 cells. Therefore, CD44s expression is indeed critical for TGF-β1-induced EMT and stemness of prostate cancer cells.

It has been reported that SMAD3 and PCBP1 play a role in controlling CD44 alternative splicing [25]. TGF-β1 and

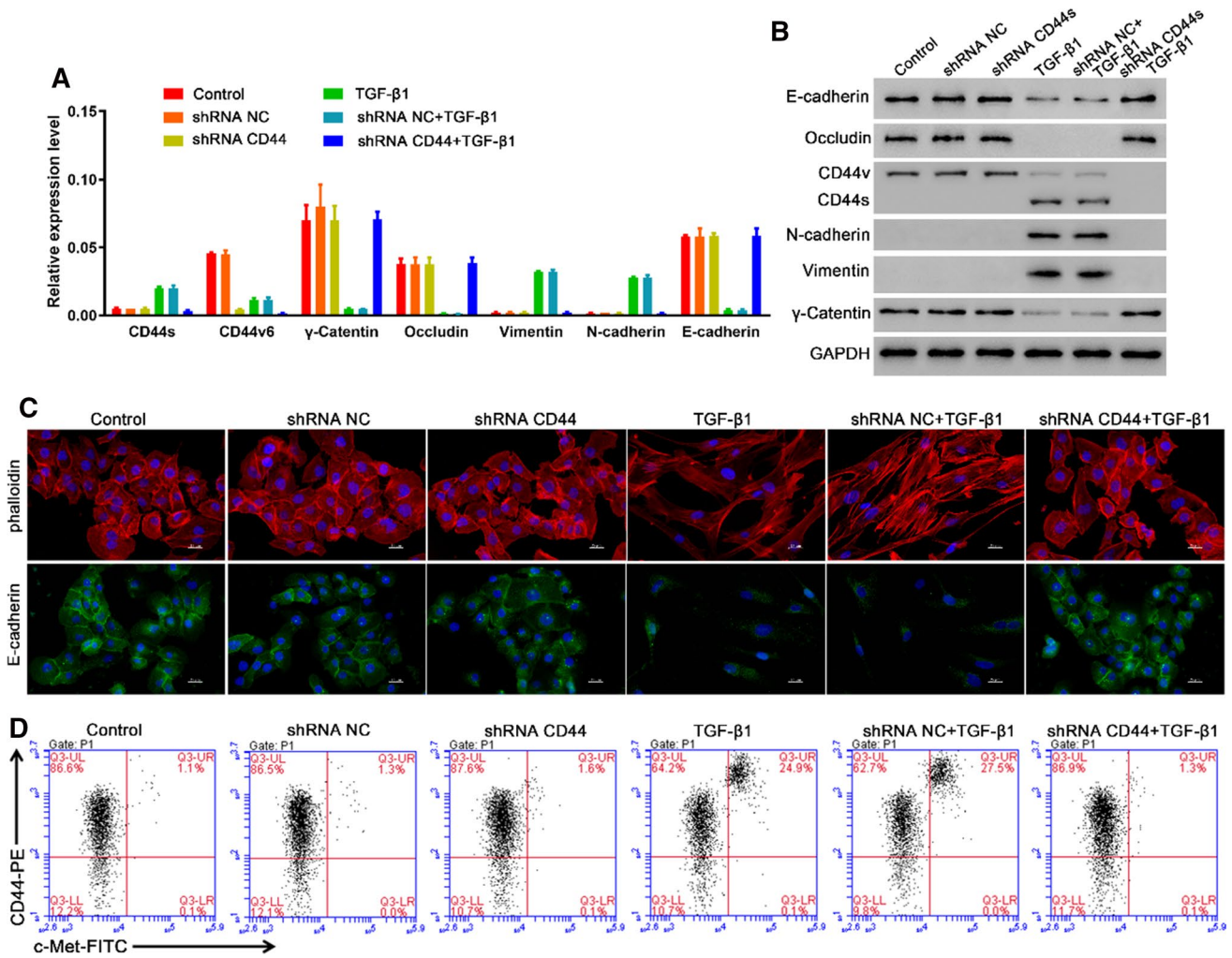


Fig. 2 Downregulating CD44 expression suppressed TGF-β1 treatment induced EMT and stem cell marker expression in prostate cancer cells. **a** DU-145 cells were mock transfected or stably expressed CD44 shRNA or negative control shRNA (NC), and then they were treated with TGF-β1 as in Fig. 1b, the mRNA expression of CD44 and EMT markers was quantified by real time-PCR. While TGF-β1 treatment induced a switching from CD44v to CD44s, upregulated mesenchymal markers vimentin and N-cadherin expression, downregulated the epithelial markers E-cadherin, occludin and γ-catenin expression, shRNA knockdown of CD44 reversed the phenotype. **b** Cells were similarly treated as in Fig. 2a, protein expression of CD44 and EMT markers were measured by western blot. Similar changes of protein expression were observed as in (**a**). **c** Cells were treated the same as above, stained with anti-E-cadherin antibody and phalloidin, and then images were taken using confocal microscopy. Scale bar: 50 μm. **d** Cells were treated the same as above, fixed and stained with anti-CD44 antibody and anti-c-Met antibody, and then analyzed by flow cytometry

EGF treatment-induced signaling event led to the phosphorylation of Threonine 179 of SMAD3, which enhanced the interaction between PCBP1 and SMAD3 on CD44 pre-mRNA. It then caused the alternative exon usage and a switching from CD44v to CD44s expression. To test the possible role of SMAD3 and PCBP1 in our experiment system, we applied RNAi to knockdown these two splicing factors. As shown in Fig. 4a & c, SMAD3 shRNA treatment led to the reduced expression of SMAD3 and CD44s in TGF-β1-induced cells, while the expression of CD44v was increased, which in turn reversed the EMT process, as indicated by the increased expression of epithelial marker E-cadherin and decreased expression of the mesenchymal marker vimentin. The percentage of stem cell marker positive cells was also reduced after knockdown SMAD3 in TGF-β1 treated cells (Fig. 4e). All these data indicated that SMAD3 was required for TGF-β1-induced CD44 alternative splicing, EMT and stem cell generation in prostate cancer cells, which is consistent with the previous finding. In Fig. 4b,d and e, we presented the data from experiments in which PCBP1 was knocked down by RNAi. PCBP1 knockdown led to increased expression of CD44s and decreased expression of CD44v in prostate cancer cells without TGF-β1 treatment. The EMT markers and the percentage of stem

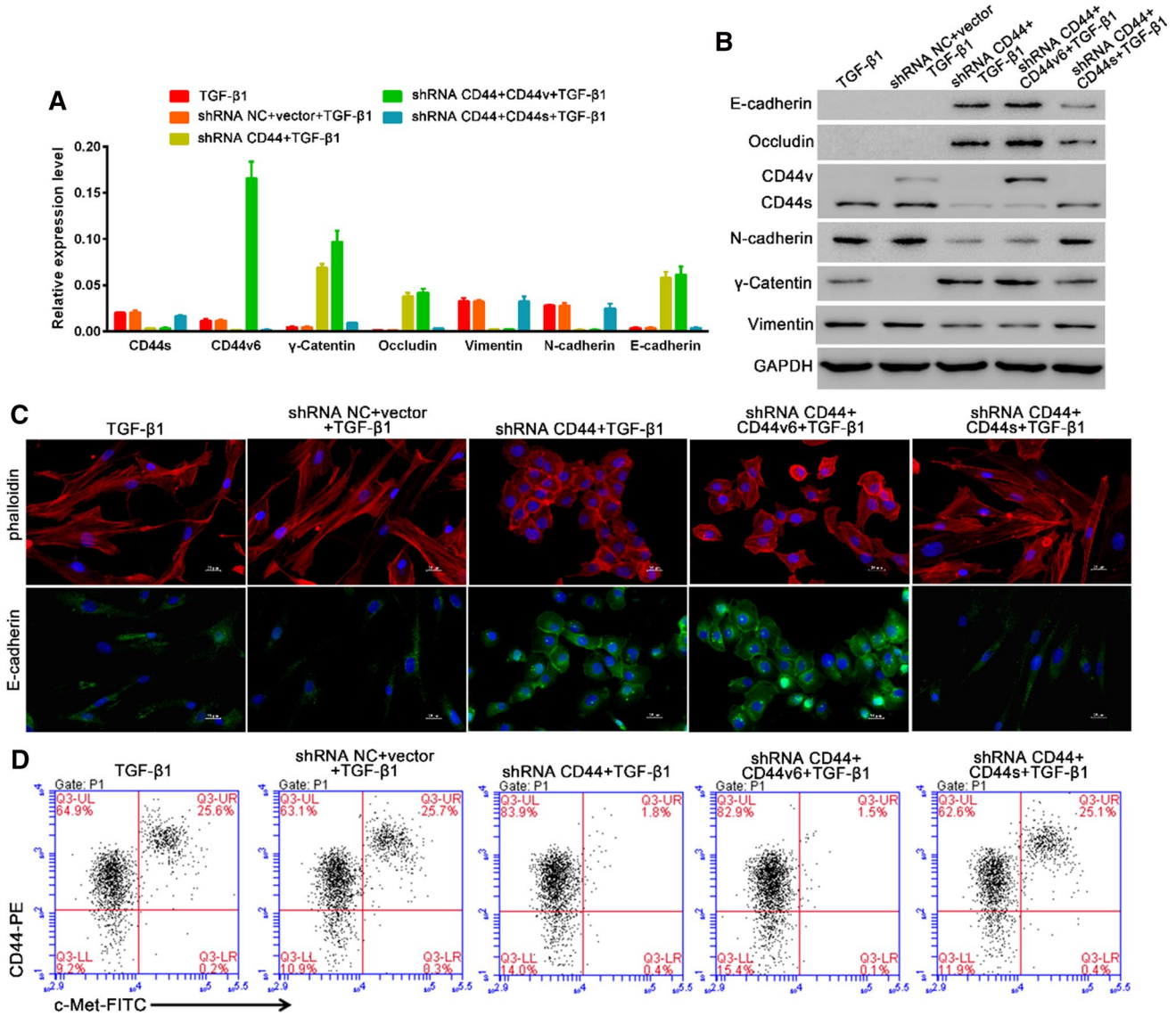


Fig. 3 Overexpression of CD44s but not CD44v6 in CD44 knockdown cells rescued the TGF- induced EMT and stem cell marker expression in prostate cancer cells. Cells stably expressed CD44 shRNA or negative control shRNA (NC) were transfected with CD44s or CD44v6 RNAi-resistant expression plasmid, and treated with TGF-β1, and then the expression of CD44 and EMT makers at the mRNA level (**a**) or protein level (**b**) were measured. **c** Cells were stained with anti-E-cadherin antibody and phalloidin, then images were taken under confocal microscopy. Scale bar: 50 μm. **d** Cells were stained with anti-CD44 antibody and cancer stem cell marker c-Met antibody, then analyzed by flow cytometry

cell marker positive cells were also correlatively changed in those cells. The data indicated that SMAD3 and PCBP1 was important in mediating the effect of TGF-β1 treatment and knockdown of PCBP1 was sufficient to mimic TGF-β1 treatment to induce CD44v to CD44s switching and to promote EMT and stemness of prostate cancer cells.

We next performed experiments to dissect the underlying mechanisms. TGF-β1 treatment did not reduced the mRNA level of PCBP1 (Fig. 5a), but it decreased the protein level of PCBP1 in DU-145 prostate cancer cells (Fig. 5b). Treating the cells with inhibitors of different kinases downstream of TGF-β1 signaling, including the CDK inhibitor flavorpiridol , the AKT inhibitor VIII and the MEK1/2 inhibitor U0126, reversed the EMT and stem cell marker expression in DU-145 cancer cells (Fig. 5c,d, and e and Fig. S3), indicating that all of them contributed to the effect of TGF-β1 treatment. We next focused on how TGF-β1 downregulated PCBP1 expression at the protein level. We found TGF-β1 treatment enhanced the polyubiquitination of PCBP1 (Fig. 6a). Treating the cells with kinase inhibitors flavorpiridol, VIII and U0126 reduced the level of PCBP1 ubiquitination (Fig. 6b), suggesting TGF-β1 very likely induces protein

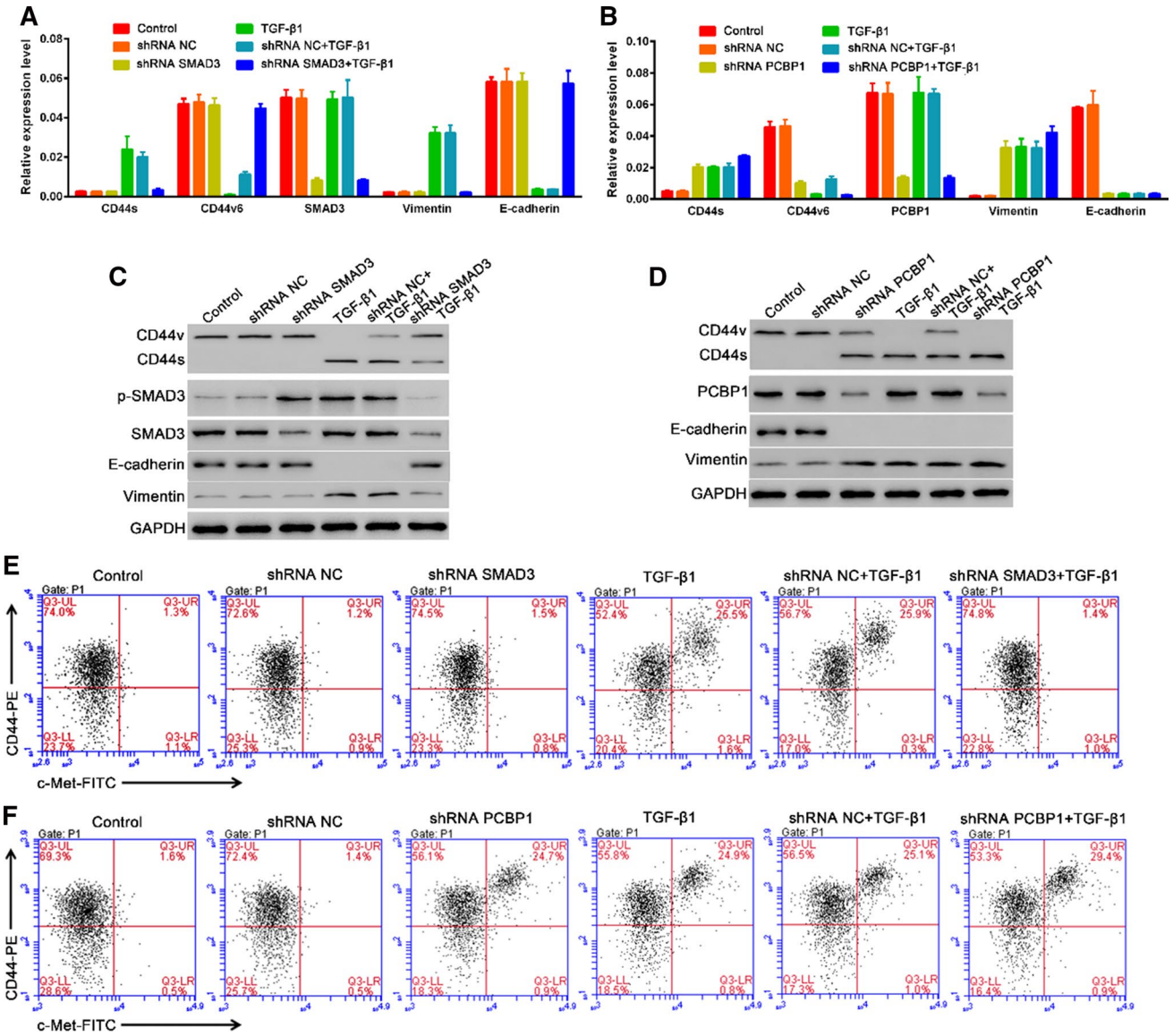


Fig. 4 Knockdown of SMAD3 inhibited and knockdown of PCBP1 mimicked the TGF-β1 induced phenotype in prostate cancer cells. Cells were transfected with SMAD3 specific or negative control shRNA, treated with TGF-β1, then the expression of CD44 and EMT makers at the mRNA level (**a**) or protein level (**c**) were measured. Cells were transfected with PCBP1 specific or negative control shRNA, treated with TGF-β1, then the expression of CD44 and EMT makers at the mRNA level (**b**) or protein level (**d**) were measured. **e** Cells were treated SMAD3 shRNA and TGF-β1, stained with anti-CD44 antibody and anti-c-Met antibody, and then analyzed by flow cytometry. **f** Cells were treated with PCBP1 shRNA and TGF-β1, stained with anti-CD44 antibody and anti-c-Met antibody, and then analyzed by flow cytometry

phosphorylation and PCBP1 ubiquitination to decrease PCBP1 protein level, and then causes CD44 switching and prostate cancer cell EMT and CSC generation.

To test the role of CD44s in promoting tumor progression, we either knockdown CD44 or overexpressing CD44s or CD44v in prostate cancer cells and then performed cell proliferation, migration, and invasion assays. Knockdown CD44 reduced the cell proliferation, migration, and invasion capabilities (Fig. 7a–d). When CD44s was overexpressed together with shRNA, the cell proliferation, migration, and invasion capacities were all rescued, while overexpressing CD44v did not have an effect (Fig. 7a–d). To confirm the role of CD44s in tumor metastasis, we injected the same set of cells into mice, and the tumor metastasis and tumor growth were evaluated. shRNA knockdown of CD44 significantly decreased the number of lung metastasis, overexpressing CD44s but not CD44v reversed the phenotype (Fig. 7e and f). CD44s and CD44v expression was validated in the lung metastasis randomly selected from each group (Fig. S4). The effect on tumor growth by these manipulations was

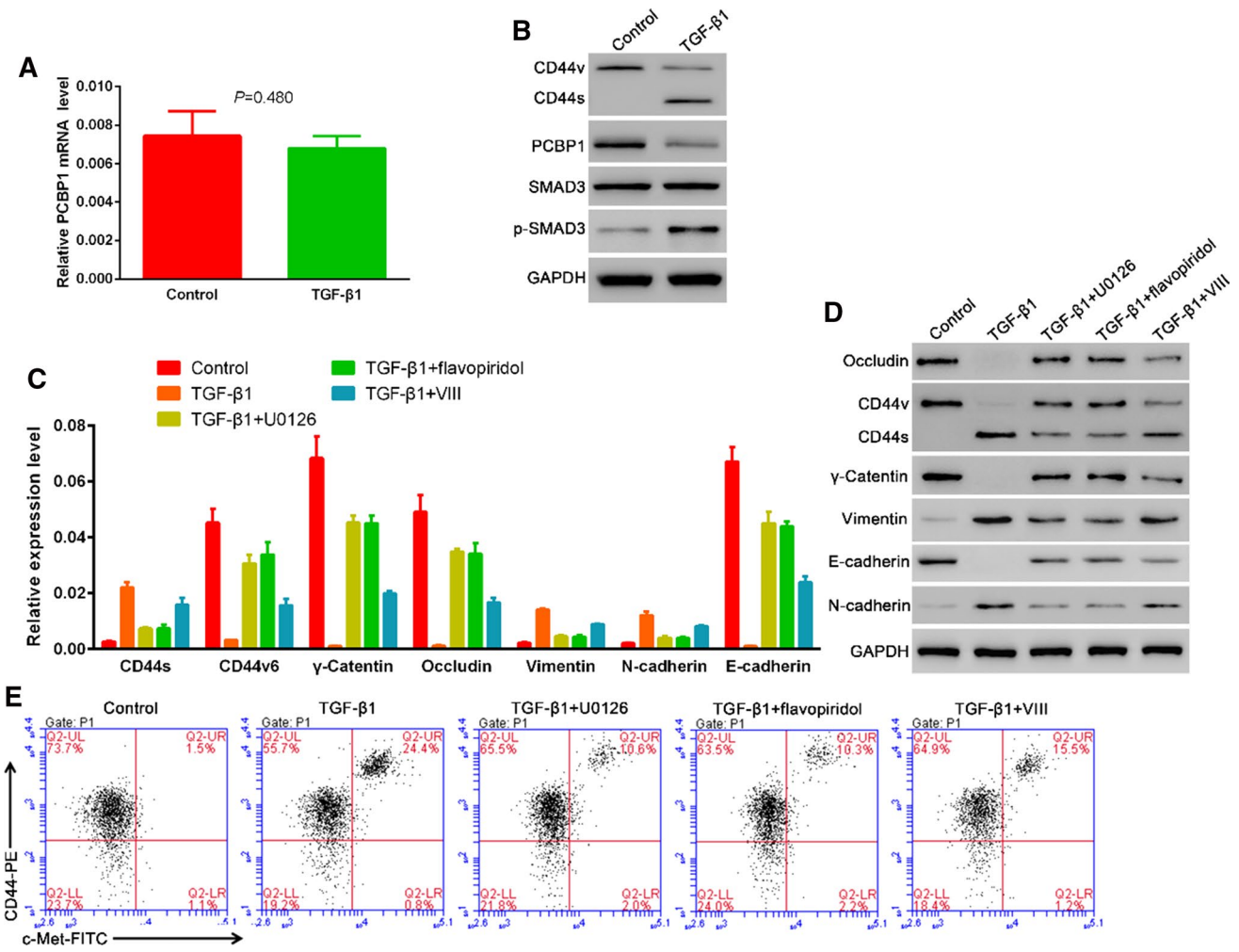


Fig. 5 TGF-β1 treatment reduced PCBP1 expression at the protein level and multiple kinase activities down stream of TGF-β1 signaling were required for TGF-β1 induced EMT and cancer stem cell maker expression in prostate cancer cells. **a** PCBP1 mRNA level was quantified by real time-PCR. There was no difference between untreated and TGF-β1 treated cells. **b** PCBP1 protein expression were measure by western blot. It was reduced after TGF-β1 treatment. **c, d**, **e** Cells were treated with kinase inhibitors U0126, flavopiridol and VIII in the presence of TGF-β1, and the expression of CD44 and EMT makers at the mRNA level (**c**) or protein level (**d**) was measured. While TGF-β1 induced a switching from CD44v to CD44s and from epithelial marker to mesenchymal marker expression, treatment by all three kinase inhibitors reversed the phenotype. When cells were stained with CD44 and c-Met antibody, and measured by flow cytometry (**e**), kinase inhibitors also suppressed TGF-β1 induced c-Met expression on the cell surface

similar. While knockdown CD44s significantly inhibited the tumor growth, overexpressing CD44s but not CD44v reversed the phenotype (Fig. 7g and h). All these data proved the important role of CD44s in promoting tumor progression in vivo.

Finally, we examined the clinical samples to verify the correlation between CD44 expression and tumor progression. CD44s and CD44v6 expression was significantly higher in tumor tissues than the adjacent normal tissues, and the CD44s expression level further increased in higher grade tumor tissues (Fig. 8a–d). Furthermore, the CD44s expression level was positively correlated with the metastasis marker N-cadherin in tumor tissues (Fig. 8e), while the CD44v6 expression level was positively correlated with E-cadherin in tumor tissues (Fig. 8f). Taken together, we reported a mechanism by which TGF-β1 treatment induces CD44s expression and confirm its importance in tumor metastasis and progression in human prostate cancers.

Discussion

CD44 has been studied in tumor biology for decades since the finding of its overexpression in many different types of tumors. Originally identified as a receptor for hyaluronic acid (HA) and lymphocyte-homing receptors, CD44 plays a role in lymphocyte homing and migration. Due to the similarities between immune cell migration and tumor

metastasis, studies have been focused on the role of CD44 in tumor migration and invasion, two critical factors of tumor metastasis.

More recently, CD44 was shown to directly link to EMT and cancer stem cell generation. Variant CD44 isoforms were found to be heterogeneously expressed in different tumor subtypes that correlates with EMT and cancer stem cell markers [26], implying that CD44 alternative splicing could be another important regulatory mechanism in EMT and CSC generation. Indeed, it has been reported that TGF-β1 activates SMAD3 in a non-canonical pathway to interact with PCBP1, which in turn interfered with PCBP1-mediated CD44 splicing [21]. Such a mechanism is critical for TGF-β1 to induce EMT and promote tumor progression. Several other mechanisms altering CD44 splicing in various types of tumor cells were also reported, suggesting that altered CD44 splicing is common in tumors. Therefore, it is of paramount importance to dissect the mechanisms, not only for a better understanding of the differential role of CD44 variants in different stages of tumor biology, but also for potential new ideas and approaches to fight against cancer.

We previously reported that TGF-β1 treatment increased the expression of CD44s and suppressed the expression of CD44v in prostate cancer cells, we also found PCBP1 could regulate CD44 splicing in prostate cancer cells [13]. In this paper, using DU-145 prostate cancer cell as a model system, we further showed that: (1) TGF-β1 treatment induced CD44 isoform switching correlate well with EMT and stem cell marker expression; (2) TGF-β1 induced upregulation of CD44s is responsible for EMT and stem cell marker expression, since RNAi knockdown of CD44 abrogated the expression of these markers, co-expressing CD44s but not CD44v was able to rescue their expression; (3) SMAD3 is required for TGF-β1 induced CD44 isoform switching and enhanced EMT and stemness in prostate cancer cells; (4) TGF-β1 induces PCBP1 polyubiquitination and degradation, which is a new mechanism for TGF-β1 induced CD44 isoform switching and tumor progression, RNAi knockdown of PCBP1 is able to mimic the activity of TGF-β1; and finally (5) CD44s promotes prostate cancer cell proliferation,

Fig. 7 CD44s but not CD44v enhanced prostate cancer cell proliferation, migration, invasion, metastasis, and tumor growth. DU-145 cells were transfected with negative control shRNA or CD44 specific shRNA in combination with CD44s or CD44v expression plasmid, then they were analyzed for cell proliferation (**b**), migration (**a** & **c**), and invasion (**a** & **d**) capacities in vitro, as well as tumor growth (**g** & **h**) and metastasis (**e** & **f**) in vivo. Knockdown CD44 reduced the cell proliferation, migration, and invasion capabilities (**a**, **b**, **c** & **d**), and reduced tumor metastasis and growth in vivo (**e**, **f**, **g** & **h**), expressing CD44s but not CD44v reversed the phenotypes ▶

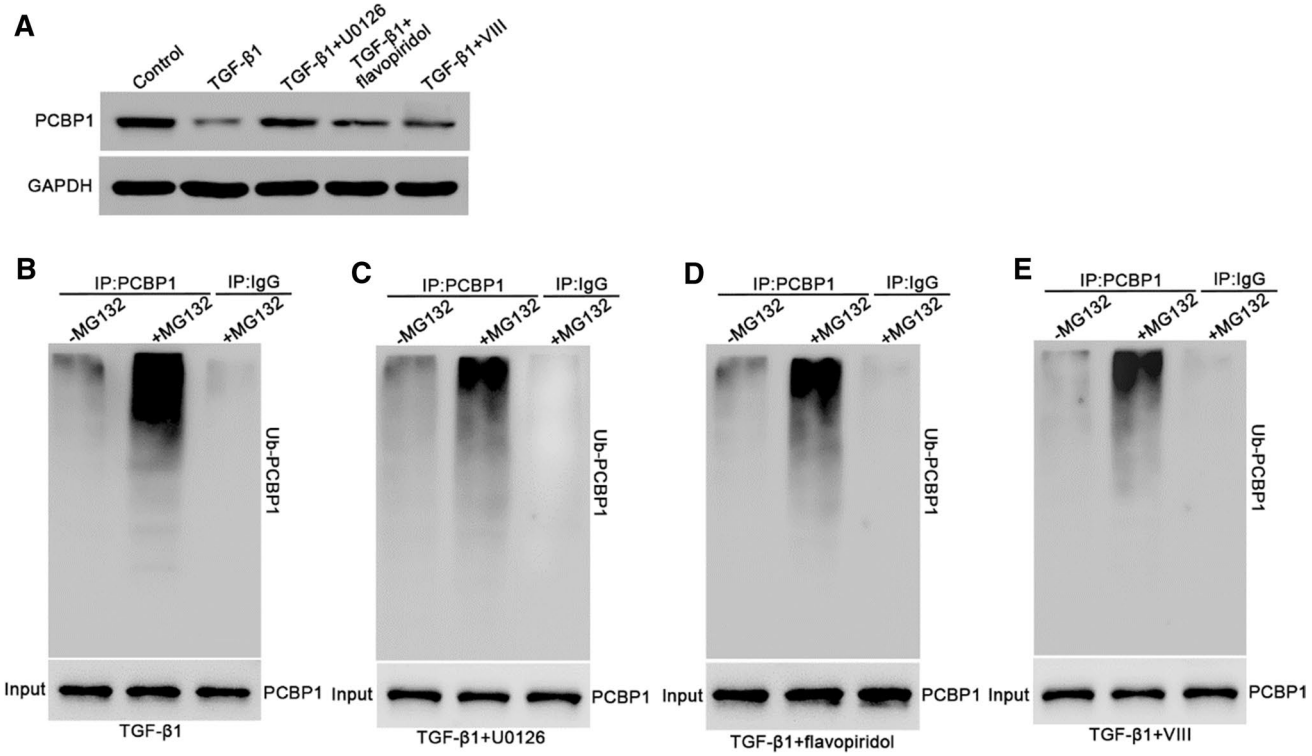
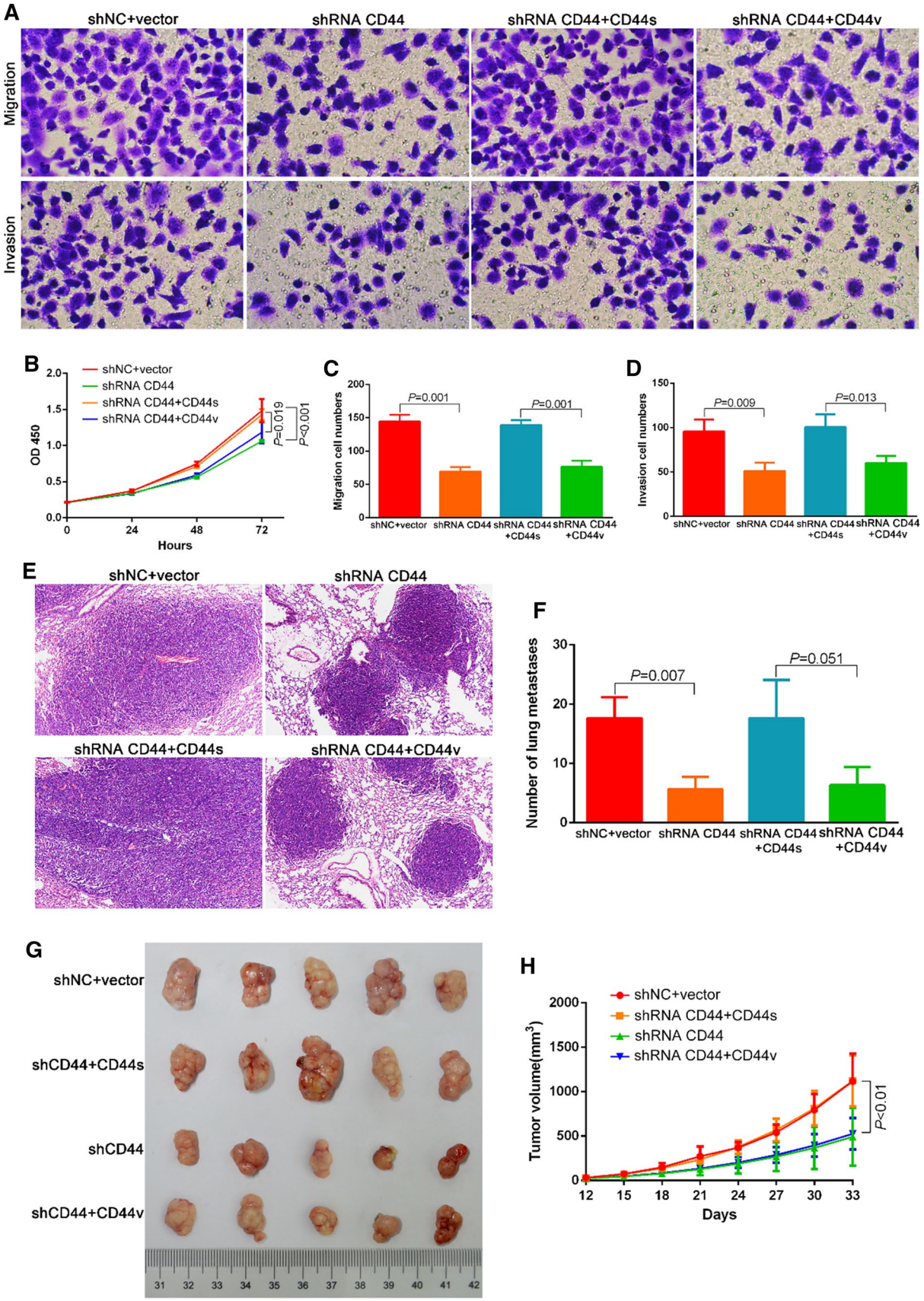
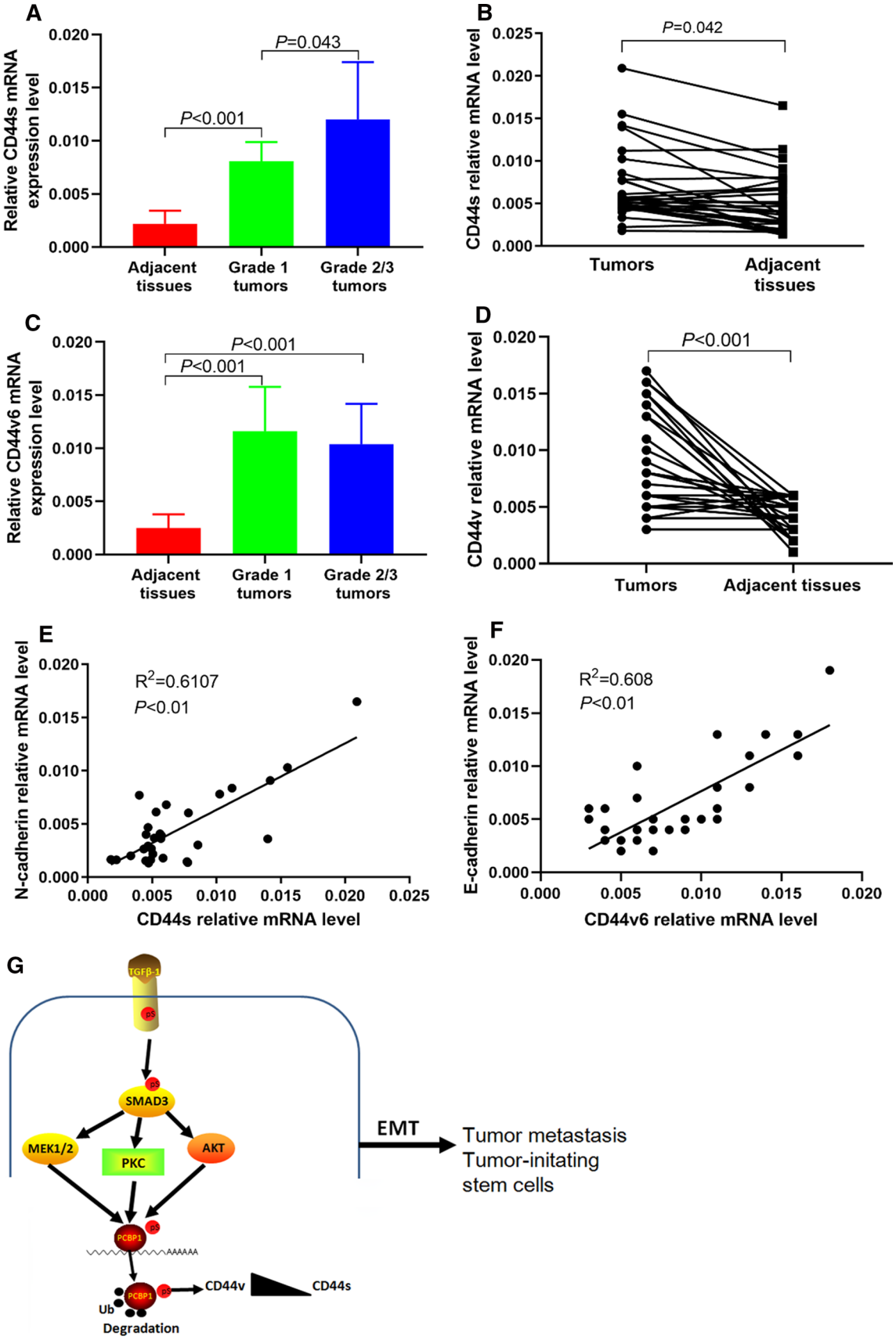


Fig. 6 TGF-β1 induced ubiquitination of PCBP1, kinase inhibitors suppressed this effect. **a** Cells were treated with kinase inhibitors U0126, flavopiridol and VIII in the presence of TGF-β1, and then PCBP1 protein expression was measured. **b** Cells were treated with TGF-β1 together with proteasome inhibitor MG132 to prevent protein degradation, PCBP1 protein was immunoprecipitated and its ubiquitination level was measured by western blot. **c**, **d**, & **e** similar experiments were performed after kinase inhibitor treatment


A
shNC+vector
shRNA CD44
shRNA CD44+CD44s
shRNA CD44+CD44v
Migration
Invasion
B
shNC+vector
shRNA CD44
shRNA CD44+CD44s
shRNA CD44+CD44v
OD 450
Hours
P=0.019
P<0.001
C
Migration cell numbers
P=0.001
P=0.001
D
Invasion cell numbers
P=0.009
P=0.013
E
F
Number of lung metastases
P=0.007
P=0.051
G
shNC+vector
shCD44+CD44s
shCD44
shCD44+CD44v
H
Tumor volume(mm3)
Days
P<0.01


A
Relative CD44s mRNA expression level
0.020
0.015
0.010
0.005
0.000
P=0.043
P<0.001
Adjacent tissues
Grade 1 tumors
Grade 2/3 tumors
B
CD44s relative mRNA level
0.025
0.020
0.015
0.010
0.005
0.000
P=0.042
Tumors
Adjacent tissues
C
Relative CD44v6 mRNA expression level
0.020
0.015
0.010
0.005
0.000
P<0.001
P<0.001
Adjacent tissues
Grade 1 tumors
Grade 2/3 tumors
D
CD44v relative mRNA level
0.020
0.015
0.010
0.005
0.000
P<0.001
Tumors
Adjacent tissues
E
N-cadherin relative mRNA level
0.020
0.015
0.010
0.005
0.000
R2=0.6107
P<0.01
0.000
0.005
0.010
0.015
0.020
0.025
CD44s relative mRNA level
F
E-cadherin relative mRNA level
0.020
0.015
0.010
0.005
0.000
R2=0.608
P<0.01
0.000
0.005
0.010
0.015
0.020
CD44v6 relative mRNA level
G
TGFβ-1
pS
SMAD3
MEK1/2
PKC
AKT
PCBP1
AAAAAA
CD44v
CD44s
Ub
Degradation
EMT
Tumor metastasis
Tumor-initating
stem cells

◂**Fig. 8** CD44s expression was positively correlated with tumor progression and tumor invasion markers. **a** CD44s mRNA expression was detected in Grade 1 tumors ($n = 10$), Grade 2/3 tumors ($n = 20$) and adjacent normal tissues ($n = 30$). CD44s mRNA expression was increased with the advance of prostate cancer. **b** CD44s mRNA expression was higher in tumor tissues than adjacent normal tissues ($n = 30$). **c**, **d** CD44v6 mRNA expression was increased in prostate cancer. **e** CD44s mRNA level was positively correlated with the mRNA expression of N-cadherin. **f** CD44v mRNA level was positively correlated with the mRNA expression of E-cadherin. **g** A diagram summarizing the findings of this paper.

migration, and invasion both in vitro and in vivo. Together with the evidence of the correlation between total CD44 expression and prostate cancer progression in clinical samples (Fig. 8a–d), our data proved an important role of switching from CD44v to CD44s in prostate cancer progression, and also revealed a new mechanism in controlling CD44 alternative splicing. However, because no antibody is available to discriminate between the two CD44 isoforms (CD44s and CD44v), the clinical significance of CD44v and CD44s in prostate cancer is to be explored in the future.

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Author contributions ZW, YBC and QC conceived the project, designed the experiments, and wrote the manuscript. QC, MG and ZKC performed and interpreted the majority of the experiments. HZ, SCS, CL and MZ performed experiments and analyzed the data. ZW and YBC supervised the project.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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